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# Human Prenatal Small Intestine Cells as a Valuable Source of Stem Cells and Epithelial Cells: Phenotypic and Functional Characterization

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**ABSTRACT:** As the fastest self-regenerating organ, the intestinal epithelium serves primarily to absorb nutrients and minerals, while its other distinct functional characteristics include the capacity to proliferate and replicate many lineage precursors. Currently, in vitro studies using small intestine cells are hindered by the lack of a standard cell culture model.

Here, we characterize primary human prenatal small intestine epithelial cells (HPIEC). The data demonstrate that HPIEC express markers indicative of stem cells (*NOTCH1*, SOX9, *OLFM4*, LGR5), epithelial cells (CK18, *CDH1*), and immunological and entero-endocrinal cells (*DEFA5*, GPR-120, GLP-1). More importantly, HPIEC are shown to secrete GLP-1 and lysozyme, and express sucrase–isomaltase, maltase–glucoamylase, and drug transport function that is cyclosporin A repressible, which demonstrates functionality. These data indicate that HPIEC are a heterogeneous cell population that contains stem cells and epithelial cells that may be valuable for various functional, developmental, and pharmacologic studies.

**KEYWORDS:** in vitro, small intestine cells, characterization, stem cells

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## Introduction

The small intestine plays a crucial role in digestion and nutrient absorption, in constant competition with a plethora of microbes and their toxins which are part of the normal intestinal flora. As the physical boundary between the outside and inside environments, the intestinal epithelium is derived from stem cells. These stem cells reside at the base of the crypt and provide all the lineages of epithelial cells, as they continuously divide, differentiate, and migrate toward the apex of the villus from where they slough off.[1,2] The epithelium of the adult mammalian intestine is in constant dynamic interaction with its underlying mesenchyme controlling direct progenitor proliferation, lineage commitment, terminal differentiation, and ultimately cell death.[2]

Small intestine epithelial cells are potentially a suitable model for researching inflammatory bowel disease, colitis, intestinal cancer, intestinal infections and more importantly, toxicity and drug discovery. Although recent research has described a variety of culture methods,[3–7] no simplified long-term culture system has been established from human prenatal intestinal tissue that maintains basic crypt–villus physiology and metabolic functionality in the differentiated state. The majority of studies are not performed on primary intestinal epithelial cells that have been derived directly from human intestinal tissue. This is in part due to limited access to viable human intestines and the complexity to isolate, purify, and propagate primary human intestinal epithelial cells. As an alternative, commercially available intestinal epithelial cell lines such as Caco-2 and HT29 are used.[8–10] Caco-2, which is one of the most widely used and best described intestinal cell models, is derived from a neoplastic tumor of the large intestine of a 72-year-old man.[11] Although these cells could be useful for studies on cell growth, the data obtained from studies on proliferation and ion transport do not necessarily support the results from the experiments performed with primary human intestinal epithelial cells.[12] Since the use of

these cell lines as tools has limitations,[2,5,9,10] efforts have been directed at developing alternative sources of human intestinal epithelial cells that maintain their functions and are easy to work with. Although culture systems of human intestinal cells have been described,[2–9] an adequate and easily obtainable primary cell culture model that is characterized and demonstrates functionality does not exist. The ideal primary cell model would contain the complete flora of cells normally found in the small intestine, be reproducible, expandable, and functional in vitro.

Here, we present phenotypic, molecular biological, and functional characterization of primary human prenatal small intestine epithelial cells (HPIECs) in vitro. These small intestine cells are a heterogeneous population that is easily expandable, possesses reproducible phenotypic properties and may possibly evade the immune response as is the case in fetal cell microchimerism,[13] which would be suitable for allogeneic transplant. HPIEC express stem cell markers, LGR5, SOX9, *NOTCH1*, and *OLFM4*;[14,15] mature epithelial cell marker, CK18; and innate immune marker, *DEFA5*. HPIEC maintain the dynamic balance between proliferation–differentiation unchanged for several passages. HPIEC also maintain stem cells and terminally differentiated cells. In addition, the data demonstrate that HPIEC inducibly secrete peptide GLP-1 and anti-microbial enzyme lysozyme, and express P-glycoprotein (Pgp) multi-drug transporter, sucrase–isomaltase, and maltase–glucoamylase. Collectively, these properties suggest that these cells are a potential in vitro model for drug discovery and development, and possibly in cell transplantation and tissue engineering studies.

## Materials and Methods

**Cell culture.** HPIEC were isolated from 17- to 18-week-old pre-natal small intestine tissue made available from elective medical abortions, with informed consent of donors. The study and consent procedure were approved by an independent institutional review board (Ethical & Independent Review Services IRB 00007807; Study #08103–05). Cell dissociation solution, culture vessel coating solution, and epithelial pro-conditioned (D-Pro) medium were all from DV Biologics, LLC (Costa Mesa, CA). The epithelial pro-conditioned medium was generated by culturing intestinal mesenchymal cells to 80–90% confluency using DMEM high glucose (Gibco, Carlsbad, CA) and 10% fetal bovine serum (FBS; Hyclone, Logan, UT), and then switching the media to low serum, 2% FBS, to stress the cells. The conditioned media were harvested after each medium replacement. These conditioned media were then combined 50/50 with basal media containing minimum essential medium (Gibco) supplemented with insulin/transferrin/selenium (BD Biosciences, San Diego, CA), using 20 ng/ml EGF and 10 ng/ml HGF (Peprotech, Rocky Hill, NJ), 200 ng/ml choleratoxin and 40 µg/ml heparin (Sigma-Aldrich, St Louis, MO) as per manufacturer's instruction. All cells used for experiments were tested for authentication using methods described below. Primary HPIEC were seeded ($1.5–4 \times 10^4$ cells/cm$^2$) in vessels (25 cm$^2$, or 6-well plates) treated with culture vessel coating solution and cultured in epithelial pro-conditioned medium (DV Biologics). To remove dead cells and cellular debris, the medium was replaced 24 hours after seeding, so cell clusters could fully adhere to the growth surface. Afterward, medium change was performed every two days. To maximize the number of serial passages, sub-culture was performed when the cells were 80 to 90% confluent (4–8 days).

**Growth kinetics.** HPIEC were seeded in duplicates at $1.5 \times 10^4$ to $2.0 \times 10^4$ cells/cm$^2$ in 6-well plates treated with culture vessel coating solution in epithelial pro-conditioned medium. The cells were dissociated with cell dissociation solution every seven days, counted, and sub-cultured at the same seeding density. Population doubling was calculated using the formula $PD = [\log_{10}(N_1) - \log_{10}(N_0)]/\log_{10}(2)$ as previously described.[16]

**Colony forming unit assay.** HPIEC were resuspended in epithelial pro-conditioned medium and seeded onto six 10 cm dishes. One dish was seeded with $1.0 \times 10^4$ cells (control) and the remaining five dishes were seeded with $1.0 \times 10^2$ cells (experimental). The plated cells were incubated in the $CO_2$ incubator for two weeks. Culture dishes were rinsed with PBS, fixed (4% formaldehyde and 1% glutaraldehyde in PBS), stained with 1% crystal violet in 10% methanol (all from Fisher Scientific, Pittsburgh, PA), destained with water, and air dried. Colonies were quantified using Bio-Rad Quantity One v. 4.6.6 software and imaging system (Bio-Rad, Hercules, CA).

**Immunocytochemistry.** Cells were cultured in 6-well plates, fixed in phosphate-buffered 4% paraformaldehyde (PFA) and stored at 4°C. After permeabilization with 0.1% of Triton X-100 for internal antigens (Promega, Madison, WI) and blocking with 1% BSA (Sigma-Aldrich) and 10% Normal Donkey Serum (Jackson Immunoresearch, West Grove, PA), primary antibodies were applied overnight at 4°C. Cells were incubated with secondary antibody in blocking buffer for one hour at room temperature (RT). Cells were counterstained with DAPI (Molecular Probes, Carlsbad, CA) and mounted with Fluoromount-G (Southern Biotech, Birmingham, AL). Primary antibodies used were mouse anti-E-cadherin, rabbit anti-Ki67, rabbit anti-ZO1, mouse anti-CK18 (Millipore, Billerica, MA), and rabbit anti-GPCR GPR-120, mouse anti-SOX9, and rabbit anti-GPCR GPR49 (anti-LGR5) (Abcam, Cambridge, England). Secondary antibodies Alexa 488 and Alexa 594 (Molecular Probes, Carlsbad, CA) were used. As negative controls corresponding specific non-immune immunoglobulins (Santa Cruz Biotech, Santa Cruz, CA) were used for all experiments. Staining was analyzed using an Olympus IX81 inverted microscope and SlideBook 4.2 software (Intelligent Imaging Innovations, Inc.).

**PCR analysis.** RNA was directly isolated from primary or cultured HPIEC and brain tissue using the RNeasy Mini

Kit (Qiagen, Hilden, Germany). cDNA was synthesized with Superscript III (Invitrogen, Carlsbad, CA) using random hexamers and 200 ng of total RNA in a 25-µl reaction. PCR was conducted in a C1000™ Thermal Cycler (Bio-Rad, Hercules, CA) using GoTaq® Green Master Mix (Promega) and 2 µl of cDNA product for the analyses of all genes. No RT controls were included in each experiment. RNA from brain tissue was used as a control to show expression of these genes in another tissue type. PCR products were visualized by agarose gel electrophoresis. Table 1 lists PCR primer sequences used for all experiments.

**Lysozyme functional assay.** The lysozyme assay was performed according to manufacturer's protocol (Worthington Biochemical, Lakewood, NJ) with modifications. Briefly, HPIEC were incubated in triplicates for two hours in assay buffer alone DMEM high glucose (Invitrogen) plus 15% FBS (HyClone) and as applicable supplemented with 0.3 mg/ml *Micrococcus lysodeikticus*. The clarified supernatants were assayed for their ability to lyse a suspension of *M. lysodeikticus* over time, by recording the absorbance at 450 nm every four minute; a solution of 5 U/ml purified lysozyme (Worthington Biochemical) in assay buffer was processed in parallel as a control.

**GLP-1 assay.** ELISA was performed according to the manufacturer's suggested protocol for GLP-1 assay kit (Millipore). Briefly, HPIEC were cultured in duplicates in 12-well plates for four days. The cells were washed twice with a rinse buffer (PBS supplemented with $CaCl_2$ and $MgCl_2$, 2 and 1 mmol/l, respectively, and 0.5% BSA), incubated for two hours at 37°C, 5% $CO_2$, in 500 µl rinse buffer containing 0.1 mmol/l Diprotin A (Sigma-Aldrich), supplemented with glucose (5 or 25 mmol/l); or with tolbutamide and/or forskolin and isobutylmethylxanthine (IBMX) (10, 10, and 500 µmol/l, respectively). Tolbutamide, forskolin, and IBMX (all from Sigma-Aldrich) were diluted from 1000× stock solutions in DMSO. Solutions with equivalent final DMSO concentrations were used as controls for samples containing tolbutamide, and/or forskolin and IBMX. GLP-1 in clarified supernatants was quantified by ELISA using a VMax Microplate Reader (Molecular Devices, Sunnyvale, CA).

**Multidrug transporter assay.** The experiment was performed using Vybrant® Multidrug Resistance Assay Kit according to the manufacturer's suggested protocol (Invitrogen). Briefly, HPIEC and Caco-2 cells were incubated in growth medium (DMEM supplemented with 15% FBS) with 0, 0.165, 1.48, 13.3, and 120 mg/l cyclosporin A for 15 min, exposed

**Table 1.** Primer sequences used in PCR studies.

| GENE | SEQUENCE | AMPLICON SIZE |
|---|---|---|
| ***CK14*** | ACGATGGCAAGGTGGTGT | 118 bp |
| | GGGATCTTCCAGTGGGATCT | |
| ***DEFA5*** | TGAGGACCATCGCCATCCTTGCT | 226 bp |
| | TCACGGGTAGCACAACGGCCG | |
| ***GAPDH*** | CAAGGTCATCCATGACAACTTTG | 496 bp |
| | GTCCACCACCCTGTTGCTGTAG | |
| ***OLFM4*** | CCAGCTAAGAGGACAAGATGAG | 286 bp |
| | GGCAGGGAAACAGAGCAC | |
| **LGR5** | GAACATGCTCACGGGAGTCTC | 303 bp |
| | CCACCCAGCAGGGGAAC | |
| **CK18** | GGGAGCACTTGGAGAAGAAG | 229 bp |
| | GATATTGGTGTCATCAATGACCT | |
| **E-cadherin-1** | AGGAACACAGGAGTCATCAGTG | 260 bp |
| | GGGGTATTGGGGGCATC | |
| **GRP120** | GCTCTGCCTCTCTGCGTCT | 255 bp |
| | GCGGATCTGGTGGCTCTC | |
| **GLP1** | ATCATTCTCAGCTTCCCAGG | 370 bp |
| | CTCATCAGAGAAAGAACCATCAG | |
| ***NOTCH-1*** | CGCCTTTGTGCTTCTGTTC | 300 bp |
| | GGTGGTCTGTCTGGTCGTC | |
| **Sucrase Isomaltase** | TTCGCTACACCTTATTACCCTTCC | 415 bp |
| | TCTCCTTTGGCTGTGTTGTTTTC | |
| **Maltase Glucoamylase** | CCCTTCGCCTGGATGTCAC | 371 bp |
| | TGGGGGCTGGTCTCGG | |

to 0.25 µmol/l calcein acetoxymethyl ester (calcein-AM) for 15 min, washed twice with growth medium, and the fluorescence was quantified using the Bio-Rad CFX-96 RT system. The results were normalized to no cyclosporin A control.

## Results

**Propagation of HPIEC.** Since there is a current absence of a small intestine primary cell model, we took on the task to characterize small intestine cells isolated from prenatal tissue and propagate them using a conditioned media obtained from intestine-derived mesenchymal cells. The culture system described herein has been optimized specifically for the culture of small intestinal epithelial cells as multi-cellular aggregates (organoids), since previous experimentation with intestinal epithelial cells indicated that breaking down the epithelium to a single-cell suspension led to poor adherence and survival of the epithelial cells. Initially seeded as organoids (Fig. 1A), HPIEC exhibit better long-term viability in culture in comparison to starting from a single-cell suspension (personal observation, data not shown). In D-PRO medium, these organoids quickly formed a lawn of highly enriched small intestine epithelial cells (Fig. 1B). Easily sub-cultured with the use of cell dissociation solution, HPIEC maintained their phenotypical morphology after four passages or nine population doublings (Fig. 1C). HPIEC could be propagated for at least six passages or 14 population doublings without any noticeable morphological changes (Fig. 1D).

**HPIEC characterization.** To better describe HPIEC, we performed molecular and immunological characterizations using immunocytochemistry (ICC) and RT-PCR. ICC analysis demonstrates that HPIEC express division and proliferative marker Ki67 (Fig. 2A). Similar to Ki67 ICC staining, the stem cell marker SOX9 is also expressed in some of the HPIEC (Fig. 2A). This may explain why HPIEC are easily expandable. The staining of both Ki67 and SOX9 appears to be confined to the inner cells of epithelial colonies that are formed.

Interestingly, within organoids, the epithelial stem cell marker LGR-5 is also found (Fig. 2B). Our RT-PCR data support and confirm our ICC results. PCR analysis demonstrates that HPIEC express early development and stem cell markers *NOTCH1*, SOX9, *OLFM4*, and LGR5 (Fig. 2C).

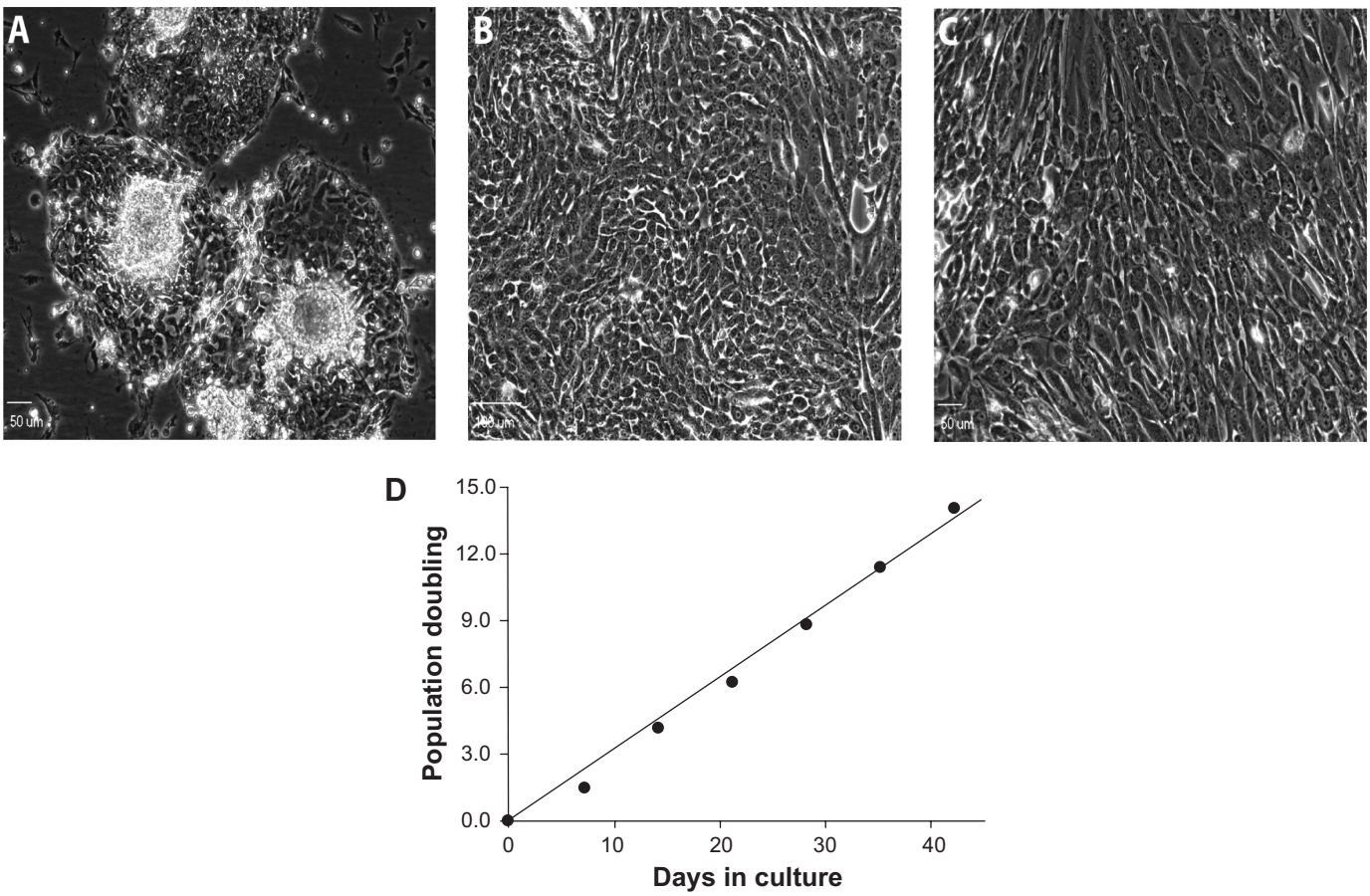


**Figure 1. Growth characteristics of HPIEC. A**) Phase contrast of freshly isolated HPIEC**.** After overnight attachment HPIEC show characteristic organoid structure of intestinal epithelial cells. **B**) Established culture of HPIEC, Passage 0 phase contrast. After 7 days in culture in epithelial pro conditioned medium, HPIEC display typical morphology of intestinal epithelial cells. **C**) Established culture of HPIEC, Passage 4 phase contrast. After over 8 population doublings in culture using epithelial pro conditioned medium, HPIEC maintain typical morphology of intestinal epithelial cells. **D**) HPIEC growth curve illustrating cumulative population doublings.

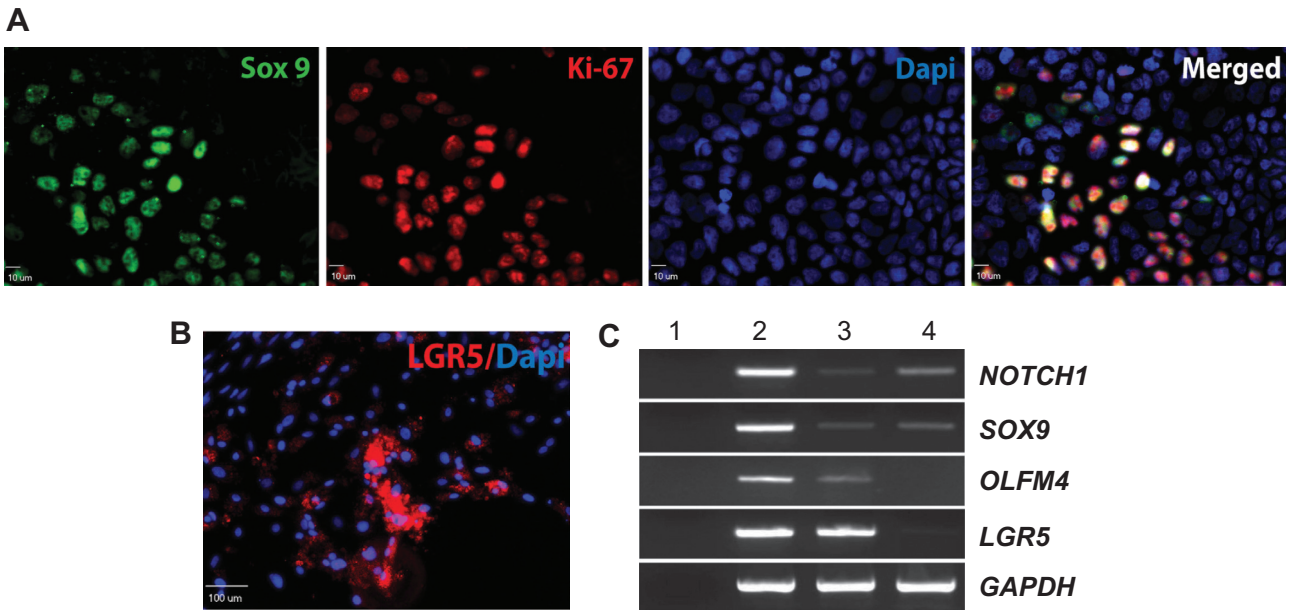


**Figure 2. Immunocytochemical staining and Molecular marker characteristics of HPIEC.** HPIEC were stained with specific monoclonal or polyclonal antibodies, counter-stained with DAPI (Blue), and visualized with fluorescence microscopy. Panel **A** illustrates staining of HPIEC with antibodies specific for SOX9 (green) and Ki67 (red). Merged images demonstrate that not all cells express SOX9 and Ki67. Panel **B** illustrates staining of LGR5 which is mostly confined to organoid structures. For panel **C** total RNA was isolated and reverse transcribed (RT); the resulting cDNA was PCR-amplified with primer pairs specific for markers of stem cells *NOTCH1*, SOX9, *OLFM4*, and LGR5. *GAPDH* was included as housekeeping marker control. **Lane 1:** No RT control; **lane 2:** Uncultured HPIEC; **lane 3:** Passage 4 HPIECs; **lane 4:** Brain tissue.

In addition, we performed colony forming unit (CFU) assays to confirm the presence of stem cells. When HPIEC were seeded at 100 cells per 10 cm dish, the data illustrate the formation of 9.6 ± 0.7 CFUs ($n$ = 5). Control that was seeded at 10,000 cells per 10 cm dish produced a lawn of cells at two weeks (data not shown). HPIEC express markers indicative of mature epithelial cells. HPIEC express adhesion of epithelial cell marker E-cadherin, tight junction associated protein marker ZO-1, the mature epithelial marker CK18, and intestinal marker G protein coupled receptor 120 (GPR-120) (Fig. 3A). Our PCR data (Fig. 3B) support and extend the ICC results. HPIEC express mature epithelial cell markers CK18 and E-cadherin, and also express the Paneth cell marker defensin-α-5 (*DEFA5*), which is thought to be involved in host defense. In addition, the markers GPR-120 and glucagon-like peptide 1 (GLP-1), which are thought to play an important role in regulating insulin, are also expressed (Fig. 3B). Indeed, our PCR analysis of epithelial cell markers (*CK-18* and *CDH1*), epithelial stem cells markers (LGR5, SOX9, and *OLFM4*), and Paneth cells (*DEFA5*) demonstrates that cultured cells in conditioned medium maintain the same gene expression profile as uncultured freshly isolated cells (Figs. 2 and 3, lane 2). These data support the notion that HPIEC contain a mixed population of stem cells and terminally differentiated cells.

**HPIEC functionality.** We observed that HPIEC secrete GLP-1 and lysozyme upon stimulation and express a repressible multidrug transporter function (Fig. 4). Using enzyme-linked immunosorbent assay (ELISA), we found that HPIEC secrete GLP-1, a protein synthesized only in the intestine by posttranslational processing of proglucagon via the L-cell.[17] HPIEC secrete GLP-1 in response to glucose in a dose-dependent manner and upon stimulation with the anti-diabetes compound tolbutamide and elevated cAMP levels that are induced by forskolin and IBMX (Fig. 4A).

Paneth cells secrete lysozyme and α-defensins, both of which have clear antimicrobial activity, and also tumor necrosis factor-alpha, and phospholipase A2.[18] For further functional testing, HPIEC were exposed to *M. Lysodeikticus*, which stimulated the secretion of lysozyme (Fig. 4B), thus confirming that the present culture system preserves the subpopulation diversity comprising Paneth cells, among others that exists in the natural environment of the small intestine. We also researched for the presence of Pgp multi-drug transporter activity in HPIEC. This was determined using the Invitrogen Vybrant MDR assay kit. This assay kit relies on the passive diffusion of the lipophilic calcein-AM, which can be carried outside the cell by a Pgp multi-drug transporter, if it is not converted to fluorescent calcein by indigenous esterases. The Pgp multi-drug transporter can be inhibited by cyclosporin A, resulting in an intracellular accumulation of fluorescent calcein. The data demonstrate that, similar to Caco-2 cells, in the present culture system, HPIEC express a Pgp multi-drug transporter activity, which is inhibited by cyclosporin A in a dose-dependent manner (Fig. 4C). Our PCR data demonstrate

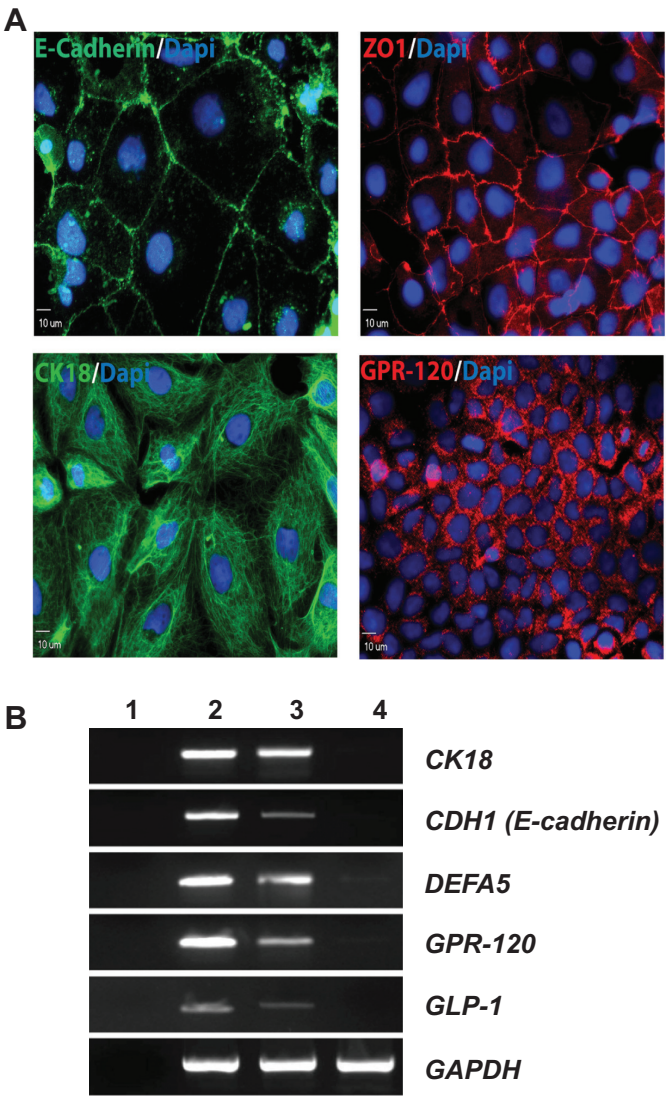


**Figure 3. Immunocytochemical staining and Molecular marker characteristics of HPIEC.** HPIEC were stained with specific monoclonal or polyclonal antibodies, counter-stained with DAPI (Blue), and visualized with fluorescence microscopy. Panel **A** illustrates antibodies specific for E-cadherin (green), ZO-1 (red), cytokeratin-18 (CK18; green) and G protein-coupled receptor 120 (GPR-120; red). For panel **B** total RNA was isolated and reverse transcribed (RT); the resulting cDNA was PCR-amplified with primer pairs specific for epithelial cells (CK18, *CDH1*), and entero-immunological and entero-endocrinal characteristics (*DEFA5*, GPR-120, GLP-1); *GAPDH* was included as housekeeping marker control. **Lane 1:** No RT control; **lane 2:** Uncultured HPIEC; **lane 3:** Passage 4 HPIECs; **lane 4:** Brain tissue.

that HPIEC express sucrase–isomaltase and maltase–glucoamylase (Fig. 4D). The expression of specific enzyme markers sucrase–isomaltase and maltase–glucoamylase is indicative of the presence of functional entrocytes. Moreover, our PCR analysis demonstrates that cultured cells in conditioned medium maintain the same expression profile as uncultured freshly isolated cells (Figs. 4D, lane 2 and 3). These results further support the notion that HPIEC are functional and a valuable source for various studies.

## Discussion

Recent advances in small intestine research illustrate the intimate interaction between intestinal stem cells and the self-renewing epithelium. This interaction can be explained by an intricate interplay among a distinct set of growth signals that are now becoming well defined.[15,19] Presently, there is no standard in vitro cell culture system for small intestine epithelial cells although several have been reported in the literature.[5–7,19] Sato et al described a small intestine cell culture system that is in a 3D matrix and requires organoid formation and maintenance. Although this system may have limitless potential, it does not appear to be a simplified in vitro model and to the best of our knowledge they have yet to test the ability of this model for specific in vitro studies such as GLP-1 secretion and multi-drug transporter assays in human cells.[19] The purpose of this study was to identify a simple cell culture system of small intestine epithelial cells that mimics the in vivo system. The system must be easy to work with and should be valuable for multiple in vitro studies. After researching current literature, we decided to use cells isolated from prenatal small intestine. HPIEC are a heterogeneous monolayer cell population that contains all the cell populations of the small intestine. Monolayer of cells is widely used throughout industry and enables rapid research and drug discovery in vitro. HPIEC exhibit stem cell self-renewal and multipotential differentiation in culture. In addition, the close proximity of Paneth cells to stem cell populations provides intestinal epithelial cells with essential signals to assemble into self-organizing structures. Thus, it is imperative to take into consideration isolation techniques that preserve close cellular interactions.[3]

The culture vessel coating solution and conditioned media of the presently described culture system provides an adequate environment supporting the growth of HPIEC. Current evidence illustrates the significant roles of the extracellular matrix (ECM) as well as cell culture media on the attachment, differentiation, and proliferation of intestinal epithelia.[3,4,20–22] Since the proliferation of epithelial crypt is *Wnt* dependent,[23,24] the method employed herein also simulates the extracellular signals found in the intestinal crypt, by utilizing a medium conditioned by mesenchymal and Paneth cells that reside in the crypt–villus niche. These cells secrete various cytokines that modulate the *Wnt* gene expression pathway similar to that expressed in vivo.[23,24]

Although the growth, maintenance, and propagation of small intestinal epithelial cells can be challenging, HPIEC are easily expandable and maintain their phenotype (Fig. 1), most likely due to the method of isolating organoids consisting of intact villi and crypt units that have a much greater capacity to adhere, survive, and maintain the polarity of epithelium.[3] Base columnar cells in select populations of SOX9 expressing cells in crypt cells were shown to produce multilineage intestinal organoids in ex vivo culture systems.[25] Indeed, HPIEC express dividing cell marker Ki67 and stem cell markers

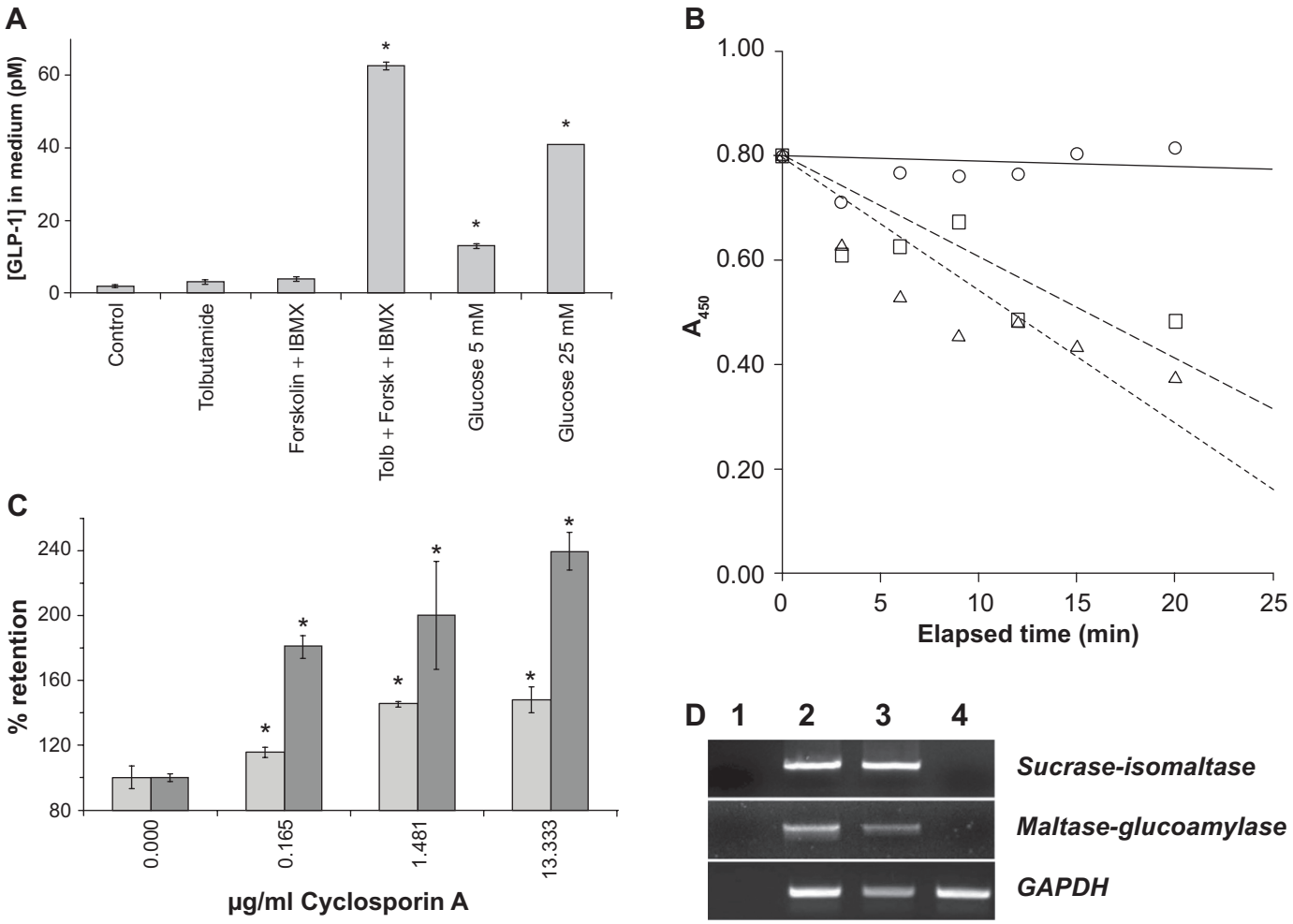


**Figure 4. Functional characterization of HPIEC. A) Synthesis of glucagon-like peptide 1 (GLP-1).** PD015-F cells were equilibrated in assay buffer for 2 h, then exposed to tolbutamide, or forskolin and isobutylmethylxanthine (IBMX), or tolbutamide, forskolin and IBMX, or 5 mM glucose, or 25 mM glucose as indicated for 2 h. GLP-1 in the clarified supernatants was assayed by ELISA, and normalized to no treatment control. **B) Synthesis of lysozyme.** PD015-F cells were incubated in assay buffer alone (O), or challenged with 0.3 mg/ml *Micrococcus lysodeikticus* (*ML*; □) for 2 h. The ability of the clarified supernatants to lyse a suspension of *ML* over time was assayed; a solution of 5 U/ml purified lysozyme served as positive control (Δ). **C) Inhibition of P-glycoprotein activity.** PD015-F cells (Lighter shade) and Caco-2 cells (Darker shade) were treated in various concentrations of cyclosporin A for 15 min, and loaded with calcein-AM (0.25 µmol/l) for 15 min; after two washes, and residual fluorescence was quantified, and normalized to no cyclosporin A control. **D) Expression of sucrase-isomaltase and maltase-glucoamylase**. Total RNA was isolated and reverse transcribed (RT); the resulting cDNA was PCR-amplified with primer pairs specific for sucrase-isomaltase and maltase-glucoamylase. **Lane 1:** No RT control; **lane 2:** Uncultured HPIEC; **lane 3:** Passage 4 HPIECs; **lane 4:** Brain tissue. Error bar: ± SEM. Asterix (*) denotes statistically significant difference over control.

SOX9, *NOTCH1, OLFM4* and LGR5 (Fig. 2). Colony forming unit assay of HPIEC suggest that approximately 9.6% of the cells may be of the stem cell origin (data not shown). The co-localization of SOX9 and the proliferation marker Ki67 in cultured cells (Fig. 2) illustrate the possibility of using human fetal cells to isolate selective stem populations capable of generating complete villi and crypt in vitro for tissue engineering purposes. Figure 2 also demonstrates the expression of LGR5, a *Wnt* targeting gene that is selectively expressed in the base of the intestinal crypt. LGR5+ stem cells are critical regulators of the cell populations at the crypt base. LGR5+ cells are unique due to their capability to form self-renewing, and multipotent organoids that can recapitulate the structure and cellular organization of intestinal epithelium from a single cell.[19]

HPIEC are a monolayer of cells. They consist of a heterogeneous population of cells found in the small intestine (Figs. 1–3). HPIEC express markers indicative of mature epithelial cells (E-cadherin, ZO-1, CK18; Fig. 3), and immunological and entero-endocrinal cell markers (*DEFA5*, GPR-120, GLP-1; Fig. 3). These data provide evidence of a natural diversity of native subpopulations of progenitor cells, stem cells, and their progeny of transit-amplifying cells.[2,3,16] HPIEC take advantage of normal stem cell properties related in organoids.[19] Future studies will aim to identify percentages of specific cell types within the HPIEC population.

Several commercially available intestinal epithelial cell lines are being used for functional studies. These commercially available cell lines are derived from transformed human

tissue such as polyps and cancerous lesions (HT-29, Caco-2, and T84),[8–10] which have their limitations that include their malignant nature and colonic origin.[5,8] HPIEC are a monolayer cell system that may be similar to native small intestine, which is confirmed by the expression of specific intestinal metabolite enzymes and transporter proteins such as ATP-binding cassette (ABC) transporters Pgp expressed by absorptive enterocytes. Since HPIEC maintain mature and specialized intestinal cell populations after isolation and propagation, these cells provide researchers with a tool to investigate digestive and absorptive capacities of normal human intestines (Fig. 4).

HPIEC have the ability to secrete GLP-1 upon stimulation with glucose or anti-diabetic and cAMP-elevating compounds (Fig. 4). GLP-1 is a secretory peptide of physiological importance. It acts as a potent incretin, specifically stimulating glucose-induced insulin release, inhibiting glucagon secretion, and modulating the ileal control machinery by slowing down gastric emptying.[17,26] As a major regulator of the insulin secretory pathway, GLP-1 has many vital physiological actions, with many great implications in recent diabetes research.[16] In addition, it has been shown to affect the central nervous system, with its neuroprotective properties and influence on learning and memory.[26] Another important feature of GLP-1 is that it plays an inhibitory role in the regulation of eating and drinking.[27] GLP-1 or a potent, long-acting natural or synthetic GLP-1 agonist, such as Exenatide (Byetta) or Liraglutide, were demonstrated to lower glycemia which has resulted in its acceptance as a treatment for patients with type 2 diabetes.[28] These data suggest that HPIEC may be a potential research model in the areas of gastrointestinal and neurological disorders, and diabetes therapy.

HPIEC secrete lysozyme upon exposure to *M. lysodeikticus* (Fig. 4) which illustrates that these cells preserve Paneth cell functional properties in the context of the intestinal epithelium. Paneth cells, which are characterized by their large eosinophilic granules of anti-microbial compounds, comprise another important subpopulation of the intestinal epithelium. They are located near the crypts that secrete, among other substances, defensin-α and lysozyme, following interaction with bacterial antigens and in response to pharmacologic stimulation.[18,29,30] It has been shown that there is decreased expression of α-defensins in Crohn's iletis.[31] Since HPIEC contain Paneth cells, this may serve as a model for research in intestinal immunity, encompassing inflammatory bowel disease, Crohn's disease, and other gastrointestinal disorders.[31]

Shown to express a Pgp-like function, repressible by cyclosporin A, using calcein-AM as the test substrate, HPIEC may be a suitable model for rapid screening of intestinal drug absorption and the detection of chemical compounds interfering with Pgp-like functions (Fig. 4C). Intestinal epithelium has the ability to transport toxic substances, such as chemotherapy drugs, which may have been taken up non-specifically by passive diffusion or in any other way into the lumen. In some cancers, these efflux functions are highly amplified and contribute to their resistance to chemotherapy. The intestinal epithelial native non-specific efflux function is a current subject in the screening process for suitable anti-cancer drugs that can circumvent the potential problem of resistance. Pgp, the most widely studied ABC transporter, functions as a biological barrier by regulating the cellular accumulation and disposition of cytotoxic and xenobiotics drugs out of cells.[32,33] In vitro and in vivo studies have demonstrated that Pgp is involved in the export of a variety of natural drugs and lies within the luminal membranes of the endothelial cells of the brain and testes, in the adrenal glands, renal proximal tubule cells, and in the apical membrane of epithelial cells of the liver, colon, and small intestine.

Responses to external stimuli have been poorly studied in most in vitro culture methods; however, the data demonstrated here show that HPIEC have the indicative feature to respond to stimuli in vitro. The expression of specific enzyme markers sucrase–isomaltase and maltase–glucoamylase is indicative of the presence of functional enterocytes (Fig. 4D). Enterocytes are responsible for many cytokines secretion, inflammatory response, and play a role in microflora adhesion proteins as well as bacterial pathogenesis.[11] Enterocytes play a key role in responses to external stimuli and interaction with the gut's bacterial flora. Thus, cytokine expressing cells are important when considering assembly of responsive architectural model of intestinal tissue.

In summary, here we have characterized a prenatal, heterogeneous primary small intestine epithelial cell which we designate HPIEC, which express several stem cell and mature epithelial cell markers. More importantly, the data illustrate that HPIEC may be an appropriate and adequate cell source for in vitro research in gastrointestinal disorders, diabetes research, drug discovery and tissue engineering studies since they contain stem cells (LGR5+), enterocytes (sucrase–isomaltase and maltase–glucoamylase expression), Paneth cells (lysozyme secretion), and enteroendocrine cells (GLP-1 secretion). Future studies will aim to quantify specific cell types in HPIEC and target growing the cells in a 3D environment or using biodegradable matrices in order to reconstitute the small intestine environment. Such studies precipitate research to help individuals suffering from intestinal cancers and possibly deficiencies of sucrase–isomaltase.

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## Author Contributions

Conceived and designed the experiments: RN, TN, RG. Analyzed the data: RN, TN, RG. Wrote the first draft of the manuscript: RN, RG. Contributed to the writing of the manuscript: TN, AK, KB. Agree with manuscript results and conclusions: RN, TN, AK, KB, RG. Jointly developed

the structure and arguments for the paper: RN, TN, RG. All authors reviewed and approved of the final manuscript.

**DISCLOSURES AND ETHICS**

This paper was subject to independent, expert peer review by a minimum of two blind peer reviewers. All editorial decisions were made by the independent academic editor. All authors have provided signed confirmation of their compliance with ethical and legal obligations including (but not limited to) use of any copyrighted material, compliance with ICMJE authorship and competing interests disclosure guidelines and, where applicable, compliance with legal and ethical guidelines on human and animal research participants.

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